'Fast' and 'slow' muscle fibres in hindlimb muscles of adult rats regenerate from intrinsically different satellite cells

J. M. Kalhovde[1], R. Jerkovic[2], I. Sefland[1], C. Cordonnier[3], E. Calabria[3], S. Schiaffino[3,4] and T. Lømo[1]

[1]*Department of Physiology, University of Oslo, PO Box 1103, Blindern, 0317 Oslo, Norway*
[2]*Department of Anatomy, School of Medicine, University of Rijeka, 5100 Rijeka, Croatia*
[3]*Department of Biomedical Sciences and CNR Institute of Neurosciences, University of Padova, 35121 Padova, Italy*
[4]*Venetian Institute of Molecular Medicine, Padova, Italy*

Myosin heavy chain (MyHC) expression was examined in regenerating fast extensor digitorum longus (EDL) and slow soleus (SOL) muscles of adult rats. Myotoxic bupivacaine was injected into SOL and EDL and the muscles were either denervated or neuromuscularly blocked by tetrodotoxin (TTX) on the sciatic nerve. Three to 10 or 30 days later, denervated SOL or EDL, or innervated but neuromuscularly blocked EDL received a slow 20 Hz stimulus pattern through electrodes implanted on the muscles or along the fibular nerve to EDL below the TTX block. In addition, denervated SOL and EDL received a fast 100 Hz stimulus pattern. Denervated EDL and SOL stimulated with the same slow stimulus pattern expressed different amounts of type 1 MyHC protein (8% *versus* 35% at 10 days, 13% *versus* 87% at 30 days). Stimulated denervated and stimulated innervated (TTX blocked) EDL expressed the same amounts of type 1, 2A, 2X and 2B MyHC proteins. Cross-sections treated for *in situ* hybridization and immunocytochemistry showed expression of type 1 MyHC in all SOL fibres but only in some scattered single or smaller groups of fibres in EDL. The results suggest that muscle fibres regenerate from intrinsically different satellite cells in EDL and SOL and within EDL. However, induction by different extrinsic factors arising in extracellular matrix or from muscle position and usage in the limb has not been excluded. No evidence for nerve-derived trophic influences was obtained.

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Corresponding author J. M. Kalhovde: Department of Physiology, PO Box 1103, Blindern, 0317 Oslo, Norway. Email: j.m.kalhovde@basalmed.uio.no

Adult skeletal muscle fibres regenerate after injury mainly or solely from activated satellite cells that reside between the sarcolemma and the basal lamina (Bischoff, 1994; Zammit *et al.* 2002). In the absence of innervation, regenerating fast-twitch EDL and slow-twitch SOL muscles of adult rats express only fast type 2 myosin heavy chains (MyHCs) in what has been called the default pathway (Esser *et al.* 1993; Jerkovic *et al.* 1997). Predominantly fast type MyHC expression also occurs in regenerating EDL and SOL if they both become reinnervated by the fast EDL nerve, whereas predominantly slow type 1 MyHC expression occurs if they both become reinnervated by the slow SOL nerve (Snoj-Cvetko *et al.* 1996*a*,*b*; Erzen *et al.* 1999). Such results demonstrate that innervation strongly affects the pattern of MyHC expression in regenerating muscle fibres but do not reveal which nerve-derived factors are responsible. Candidate factors are the different impulse patterns generated in fast EDL and slow SOL motor neurones (Hennig & Lømo, 1985) and putative trophic factors that motor neurones to fast and slow muscle fibres may release at neuromuscular junctions (Buller *et al.* 1960; Spector, 1985; Salviati *et al.* 1986; Witzemann *et al.* 1991). In addition, intrinsic differences among satellite cells in EDL and SOL muscles and in the composition of their extracellular matrices may affect the emergence of different fibre types during regeneration.

Erzen and coworkers found that rat SOL and EDL expressed essentially identical proportions of MyHC isoforms after regeneration for 3–6 months in the bed of one or the other muscle and reinnervation of both muscles by either the SOL or the EDL nerve (Snoj-Cvetko *et al.* 1996*a*,*b*; Erzen *et al.* 1999). They therefore proposed that EDL and SOL regenerate from the same multipotential myoblast stem cell population and that extrinsic factors, such as innervation, regulate the expression of the particular phenotype. Since the regenerating SOL and EDL also underwent much greater transformation between fast and slow phenotypes than observed during cross-reinnervation or electrical stimulation of mature non-injured muscles (Close, 1969; Donovan & Faulkner, 1987; Eken & Gundersen, 1988;

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Westgaard & Lømo, 1988; Ausoni *et al.* 1990; Kirschbaum *et al.* 1990; Delp & Pette, 1994), they further suggested that muscle adaptation becomes irreversibly restricted early in development. Consistent with this last idea, Pette *et al.* (2002) observed greater expression of type 1 MyHC in regenerating compared to non-regenerating EDL muscles during low frequency stimulation of the EDL nerve and suggested that chronic low frequency stimulation can induce satellite cells and/or regenerating fast rat muscle fibres to switch directly to a slow program if the stimulation is imposed sufficiently early.

The factors that determine fibre type in regenerating muscles are difficult to evaluate in experimental models such as cross-transplantation of muscles, cross-reinnervation, and chronic nerve stimulation. For example, an undetermined pattern of endogenous nerve activity will add to the pattern imposed by nerve stimulation, and the endogenous activity may itself become markedly modified by injury to the same or adjacent nerves (Hennig, 1987). Hence, the precise impulse pattern reaching the muscle in such models is unknown. Stimulation of muscles via the nerve also does not unambiguously distinguish between effects of activity pattern and putative trophic factors because the imposed foreign stimulus pattern may change the properties of motor neurones (Gallego *et al.* 1978) and hence modify the expression and/or release of putative trophic factors.

In the present work, we have tried to overcome such limitations by denervating EDL and SOL muscles as they begin to regenerate and then stimulating both muscles directly with the same stimulus pattern, using a slow tonic pattern at 20 Hz or a fast pattern at 100 Hz that roughly resembles the natural firing pattern of slow SOL or fast EDL motor neurones, respectively (Hennig & Lømo, 1985). The model of stimulating denervated muscles directly has been previously validated by showing that such stimulation can restore denervated muscle fibres to normal with respect to membrane properties (Westgaard, 1975; Lømo & Westgaard, 1976) and be comparable to self-reinnervation in restoring maximal tetanic force production (Hennig & Lømo, 1987) and to cross-reinnervation when appropriate stimulation patterns are chosen (Eken & Gundersen, 1988). Using this approach, the exact impulse pattern imposed on the muscle is known and any confounding trophic influence from the nerve is absent. We then asked if a given stimulus pattern will induce identical phenotypes in muscle fibres regenerating from satellite cells in EDL and SOL.

Methods

Adult male Wistar rats weighing 250–300 g at the beginning of the experiment were used. All operations described below were carried out under full anaesthesia with Equithesin (42.5 mg chloral hydrate and 9.7 mg pentobarbitone in 1 ml solution, 0.4 ml (100 g body wt)$^{-1}$, I.P.), as assessed by the absence of reflex muscle contractions and limb withdrawal to squeezing the skin during surgery. After the operation for the chronic experiments, the animals received Temgesic (0.3 mg ml^{-1} in 0.25 ml subcutaneously) for suppression of postoperative pain before they woke up from the anaesthesia. At the end of the chronic experiments, the animals were fully anaesthetized as above for removal of muscles. They were then given an overdose of Equithesin and killed by cervical dislocation. In a few animals, including those used initially to develop suitable technical and surgical procedures, the chronically implanted electrodes broke before the end of the experiment or the animals started to bite their toes and distal parts of the foot. Such animals were killed immediately and excluded from the material presented here.

The experiments involving chronic muscle and nerve stimulations had been inspected and permitted by the Norwegian Experimental Board and Ethical Committee for Animal Experiments and were overseen by the veterinarian responsible for the animal house. During the experiments the animals were checked daily and did not appear to suffer pain.

Surgery

In one series of experiments the sciatic nerve was cut in the thigh of one leg and its proximal end reflected and sutured to the subcutis to prevent reinnervation. The ends of two flexible, multistranded steel wires (AS 632, Cooner Sales Wire Co., Chatsworth, CA, USA) with their Teflon insulation removed were placed across the SOL or EDL, one wire on the anterior side, the other wire on the posterior side, making sure that the uninsulated segments touched muscle fibres and not tendons as they ran across the entire muscle without touching each other. On SOL, the wires ran transversely across the muscle, the posterior wire lying proximal to the anterior wire. On EDL, the wires were placed obliquely across the EDL in a proximo-distal direction, one wire on the anterior side, the other wire on the posterior side distal to the first. Thin 5 mm-wide silicon sheets (Silastic sheeting 500–1, Dow Corning Corp., Midland, MI, USA) were placed over each uninsulated electrode to avoid spread of current to tissue outside the targeted muscle. This modification substantially reduced the amount of voltage needed for effective excitation of regenerating muscle fibres.

In another series of experiments, a small capillary for slow release of TTX was inserted into the sciatic nerve where the sciatic nerve branches into fibular, tibial and sural nerves in the fossa poplitea. The capillary (i.d. = 1.12 mm), containing 30 mM TTX in 5 μl of citrate buffer, was closed at one end and tapered off at the other to an inner diameter of 28–30 μm. A soft cuff of

silicone rubber was placed around the nerve at the site of the capillary to limit the escape of TTX and raise its concentration in the nerve, as described (Reid *et al.* 2003). Uninsulated ends of two stimulating wires (see above) were then implanted to stimulate the axons to the EDL below the TTX block. The uninsulated ends were fixed with small sutures to the tissue next to the fibular nerve just above the knee without touching the nerve itself. The conduction block was complete throughout the chronic experiment as shown by lack of flexor responses to daily pinching the skin over the lateral aspect of the foot and, at the end of the experiment, by observing vigorous muscle contraction when the sciatic nerve was stimulated below the TTX block but no contraction when it was stimulated above the block.

For both experiments each insulated wire was attached by sutures to subcutaneous tissue just above their distal uninsulated parts to prevent these parts from being pulled away from SOL or EDL muscle or fibular nerve at later times. Bupivacaine (Marcaine, 0.5%, total volume 0.4–0.6 ml) was then injected at multiple sites into denervated SOL or EDL or neuromuscularly blocked EDL to induce muscle necrosis followed by regeneration. The openings in the leg were closed by sutures (subcutaneous tissue) and metal clips (skin) and the remaining lengths of the wires run under the skin to attachments to the skull by screws and dental cement and thence through a protective plastic tube to rotating contacts and stimulator above the cage, as described (Windisch *et al.* 1998).

Chronic stimulation started 3 days and continued until 10 or 30 days after denervation or sciatic nerve block by TTX and injection of bupivacaine. The muscles were then removed and frozen in isopentane cooled in dry ice and kept at −80°C until analysed. The stimulation consisted of 200 pulses at 20 Hz every 30 s or 60 pulses at 100 Hz every 60 s. The pulses were square, bipolar, lasting 0.2 ms, and generating 5–10 mA (denervated muscles) or 1–2 mA (intact nerve) in either direction. Lack of reinnervation was confirmed, using a dissection microscope, by absence of visible contraction to stimulation of the nerve just outside EDL or SOL. Regenerating unstimulated SOL and EDL muscles with 'sham' electrodes implanted were used as controls.

In situ hybridization

Type 1 (β-slow) MyHC gene-specific cDNA was prepared from rat soleus muscle RNA. Briefly total RNA was isolated using TRIzol reagent according to manufacturer's instructions (Gibco-BRL; Cergy Pantoise, France). Total RNA was reverse transcribed using the Super-Script preamplification system (Gibco-BRL, MD, USA). A 437 bp fragment of the β-slow cDNA (GenBank accession number: X15939; 5394–5831 bp) was PCR amplified (forward 5′-accggctggacgaggcaga, reverse 5′-ggtagcacaagatctactcttcattca) and subcloned into a pCRII-TOPO (Invitrogen) plasmid vector. Full length antisense cRNA probes labelled with digoxigenin were prepared using a DIG RNA labelling kit (Boehringer Mannheim Co., IN, USA) according to manufacturer's protocol.

Cryosections 10 μm thick were placed on Superfrost Plus slides (Menzel; Merck, Amsterdam, the Netherlands), dried for 2 h at 50°C, and fixed for 20 min at 4°C with 4% paraformaldehyde in diethylpyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS). Sections were rinsed 3 × 5 min in PBS, dehydrated, and stored at 4°C under 100% ethanol until further use. Slides were rehydrated and rinsed for 5 min in PBS. After digestion with 10 μg ml^{-1} of proteinase K (in 50 mM EDTA pH 8.0 and 100 mM Tris pH 7.4) for 10 min at room temperature, the sections were treated for 1 min with 0.2% glycine (in PBS with 0.1% active DEPC), re-fixed for 5 min in 4% paraformaldehyde in PBS, rinsed 3 × 3 min in PBS and acetylated with 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min at room temperature. The sections were dehydrated, air-dried and hybridized (50% formamide, 10% dextran sulphate, 1% blocking reagent (Roche; 1096176), 0.3 M NaCl, 10 mM Tris pH 7.4 and 1 mM EDTA pH 8.0) with 200 ng ml^{-1} DIG-labelled probe at 55°C overnight. The next day, sections were washed in 2 × SSC at room temperature for 10 min and then for 30 min at 55°C in 2 × SSC and 50% formamide followed by two washes for 10 min in 2 × SSC at room temperature (RT), then treated with 50 μg ml^{-1} RNase A (in 0.5 M NaCl 10 mM Tris pH 7.4, 1 mM EDTA pH 8.0) for 30 min at 37°C, washed (in 0.5 M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA pH 8.0) for 30 min at 60°C, treated with blocking solution (2 × SSC, 0.05% Triton X-100, 1% blocking reagent) for 1 h at room temperature, and incubated with sheep anti-DIG alkaline phosphatase-conjugated antibody (diluted 1 : 3000 in blocking solution) overnight at 4°C. The sections were then washed for 3 × 5 min in 1 × maleate and finally with 0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$ for 10 min. mRNA was detected using alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 180 μg ml^{-1}), and nitroblue tetrazolium chloride (NBT; 340 μg ml^{-1}) in the presence of 2 mM levamisole for 24 h under darkroom conditions. The colour reaction was terminated by rinsing the sections in 10 mM Tris pH 7.4, 1 mM EDTA pH 8.0 for 2 × 5 min at RT, and the sections were dehydrated and covered with a mixture of glycerin and gelatine.

Immunocytochemistry

Unfixed 8–10 μm-thick cryosections were used. The sections were first blocked for 30 min at room temperature with 20% normal goat serum and 1% BSA in 0.01 M PBS, then incubated with primary antibodies (BA-D5 mouse antislow myosin, or BF-B6 mouse antiemb/neon myosin

diluted in blocking buffer) for 1 h at room temperature, and after washing, with secondary antibody (Alexa 488 goat antimouse diluted 1 : 200 in blocking buffer) for 45 min at room temperature.

SDS-PAGE

Cryosections (25–30) 10 μm thick were prepared for SDS-PAGE as described (Talmadge & Roy, 1993). Gels were stained with Commassie blue G250 (Bio-rad, USA), and images captured with a Kodak imagestation 2000MM. Relative MyHC composition was analysed using the Scion Image software (Scion Corp., NIH, USA) and gel plotting macro. At least two independent measurements were performed on each sample. Values are given as means ± 95% confidence interval.

Results

Intramuscular injections of bupivacaine destroyed the muscle tissue, as shown in Fig. 1*B*, which presents a cross-section and a representative enlarged field of a SOL muscle that had been denervated and injected with bupivacaine 3 days earlier. Three days after such treatment, many muscle cells bind antibodies specific for embryonic and neonatal MyHCs and are therefore regenerating myotubes (Jerkovic *et al.* 1997). In the absence of innervation, regenerating SOL muscle fibres failed to grow

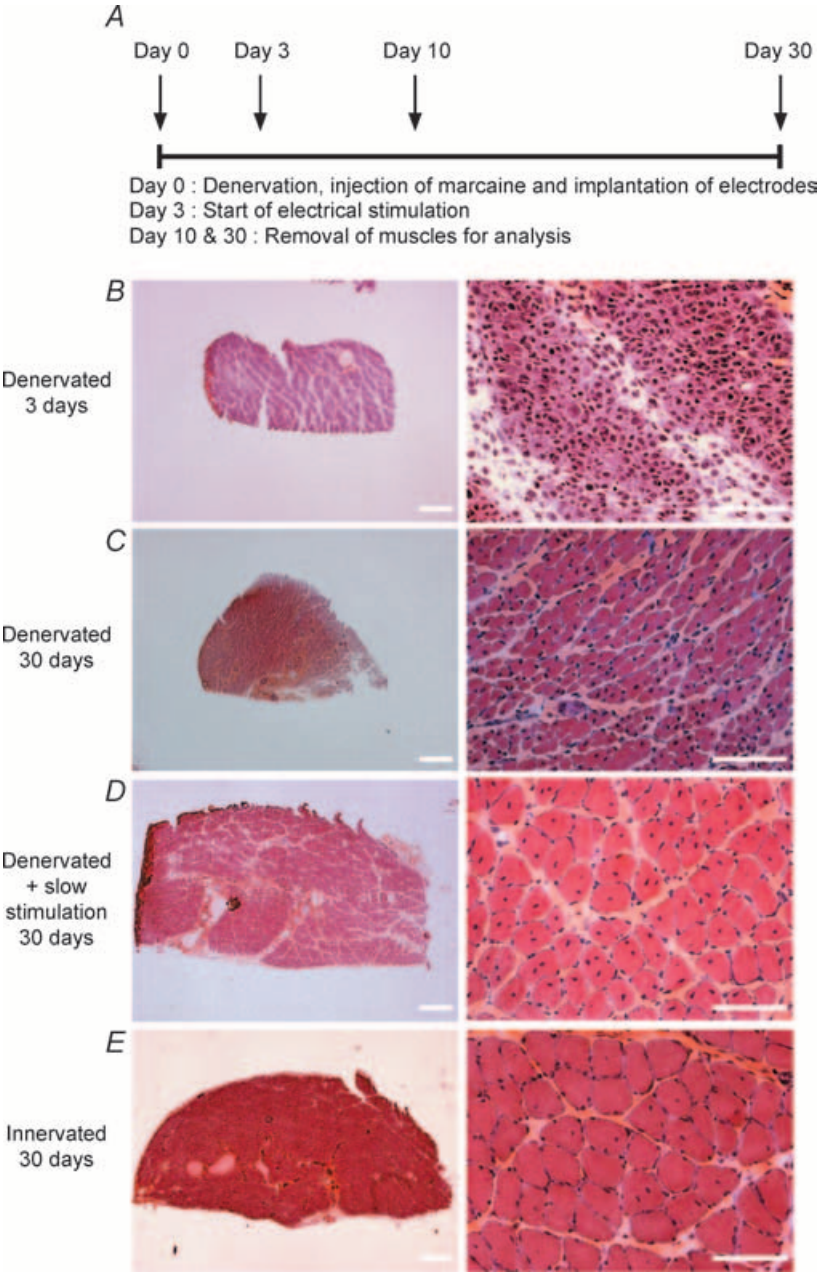

Figure 1. Morphology of regenerating SOL muscle after different treatments
The experimental protocol is shown in *A*. Representative cross-sections of muscles stained with haematoxylin, azophloxine and safran (HAS) and obtained as indicated after treatments and number of days after intramuscular injections of bupivacaine are shown in *B*–*E*. Images to the right show enlarged fields of the entire cross-sections shown to the left. Note central nuclei typical of regenerating muscle fibres and larger muscle fibres in denervated directly stimulated (*D*) and innervated (*E*) muscles compared to denervated unstimulated muscles (*C*). Scale bars: 200 μm for column to the left, 100 μm for column to the right.

normally (Fig. 1*C*). Direct muscle stimulation from day 3 counteracted this atrophy (Fig. 1*D*), as did innervation by the SOL nerve (Fig. 1*E*). Except for the presence of central nuclei, typical of regenerating fibres, essentially normal muscle structure was observed in both innervated and denervated stimulated muscles 30 days after bupivacaine injection, although the denervated and stimulated fibres tended to be smaller in diameter than the innervated fibres. The effectiveness of multiple intramuscular injections of Marcaine was examined in longitudinal sections through the muscle 3 days after the injections. In no cases did we observe intact segments of muscle fibres although the degree of invasion by mononuclear cells around and into the muscle fibres varied, most fibres showing massive invasion.

Regenerating SOL and EDL respond differently to identical stimulation

In the absence of innervation, neither SOL nor EDL expressed slow type 1 MyHC mRNA 10 days after injection of bupivacaine (Fig. 2*A* and *B*), confirming earlier reports (Jerkovic *et al.* 1997). However, if the denervated SOL was stimulated directly with the slow stimulus pattern, essentially all the fibres expressed type 1 MyHC mRNA (Fig. 2*C* and *E*). Similar type 1 MyHC mRNA expression was observed if the regenerating SOL was innervated by the SOL nerve (Fig. 2*G*).

Identical slow pattern stimulation affected the denervated EDL differently. In this case only scattered single fibres, or smaller groups of fibres, expressed slow type 1 MyHC mRNA, the rest being negative (Fig. 2*D* and *F*). In the regenerating innervated EDL, fibres positive for type 1 MyHC were also few and similarly scattered (Fig. 2*H*).

Comparable results were obtained when SOL and EDL were assayed for MyHC protein expression by immunocytochemistry. In the absence of innervation, neither SOL nor EDL displayed detectable slow type 1 MyHC protein (Fig. 3*A* and *B*), whereas slow pattern stimulation caused such expression in essentially all fibres in the SOL (Fig. 3*C* and *E*), as observed also in muscles

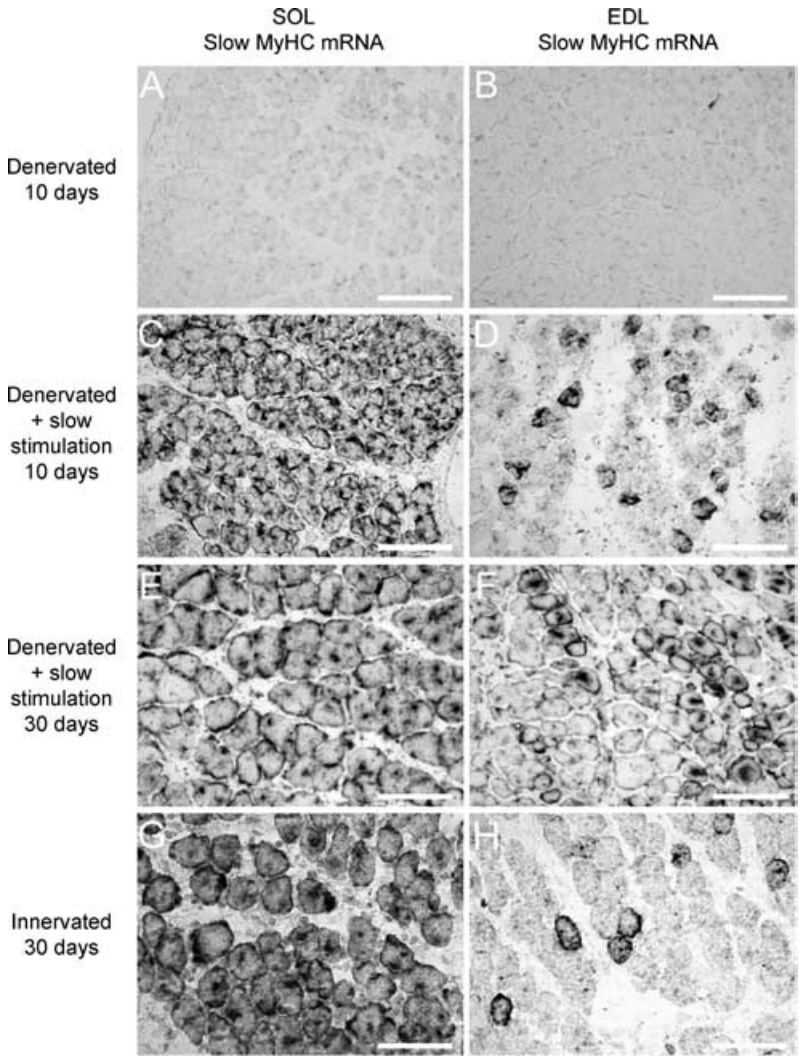

Figure 2. MyHC-slow (type 1) transcript in regenerating SOL and EDL muscles
Muscles were removed from the animals after treatments and at days after intramuscular injections of bupivacaine, as indicated. Transverse cryosections were then processed for *in situ* hybridization with MyHC type-1 specific probe. Note lack of slow type 1 transcript in denervated unstimulated SOL and EDL and presence of such transcript in all fibres of denervated stimulated or innervated SOL muscles but in only scattered single or smaller groups of fibres in denervated stimulated or innervated EDL muscles. Scale bar = 100 μm.

innervated by the SOL nerve (3G). The effect of slow pattern stimulation of the denervated EDL was different. Scattered fibres shown to express type 1 MyHC mRNA in correspondingly labelled neighbouring sections in Fig. 2, also expressed type 1 MyHC protein (Fig. 3*D* and *F*). All the fibres, however, whether positive or negative for type 1 MyHC, contained variable amounts of type 2 MyHCs (not shown). Again, the distribution of type 1 MyHC positive fibres resembled that observed in the regenerating innervated EDL (Fig. 3*H*). This result, together with the finding that both type 1 and type 2 fibres were much larger in stimulated denervated muscles compared to denervated and unstimulated muscles, indicated that all the fibres in the muscle had been effectively stimulated.

The relative amounts of adult MyHCs in regenerating SOL and EDL were quantified densitometrically from SDS-PAGE gels, representative examples of which are shown in Fig. 4*A*. The results obtained agreed with the results of *in situ* hybridization (Fig. 2) and immunochemistry (Fig. 3). Denervated and unstimulated SOL muscles expressed only type 2X and 2B MyHC (the default pathway), whereas innervated SOL muscles expressed only type 1 and type 2A MyHCs (Fig. 4*B*). Similarly, when directly stimulated with the slow stimulus pattern (20 Hz), denervated SOL muscles expressed predominantly type 1 and 2A MyHCs, particularly when the stimulation continued from day 10 to day 30, which led to a marked down-regulation of type 2X MyHC (Fig. 4*D*).

Like innervated EDL muscles, denervated unstimulated EDL muscles expressed only 2X and 2B MyHCs (Fig. 4*C*, the default pathway). No type 1 MyHC was detected in the SDS-PAGE gels from innervated EDL muscles even though a few scattered fibres in cross-sections contained both type 1 MyHC mRNA and protein (Figs 2 and 3). Presumably, the amount of slow type 1 MyHC protein in these muscles was too low for detection by gel electrophoresis and Commassie Blue staining. Unlike its effect on the denervated SOL, the slow pattern stimulation caused expression of all adult MyHC forms in the

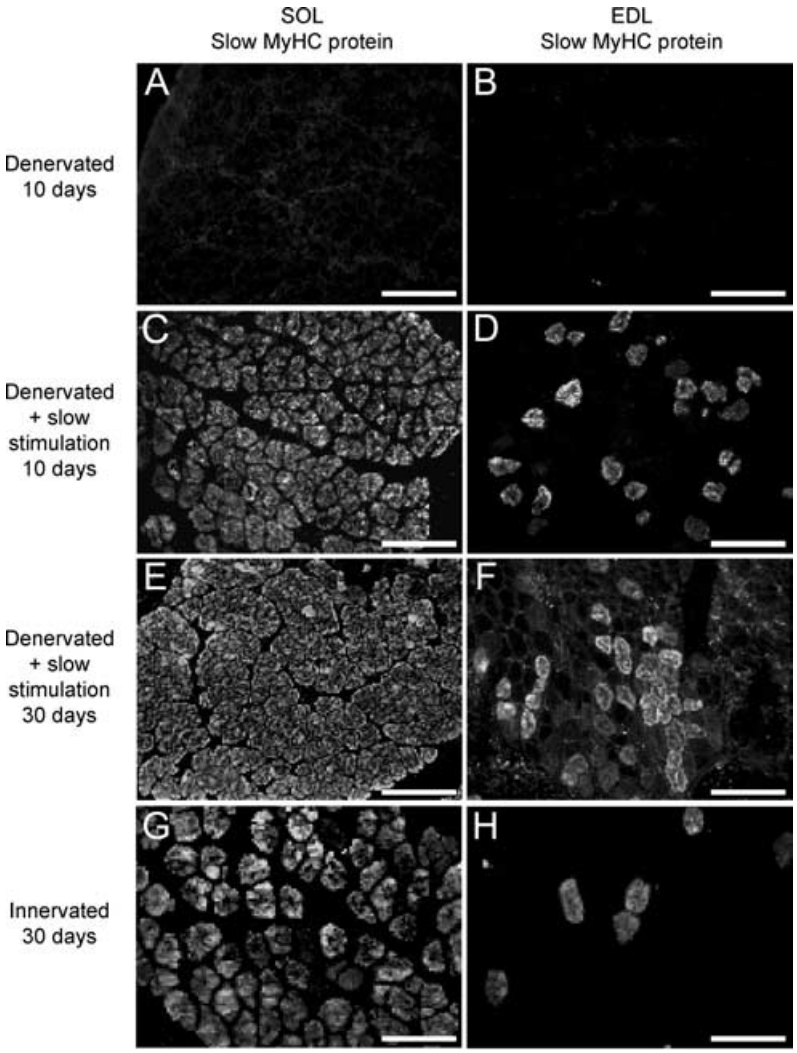

Figure 3. MyHC-slow (type 1) protein in regenerating SOL and EDL muscles
Muscles were removed from the animals after treatments and at days after intramuscular injections of bupivacaine, as indicated. Transverse cryosections were then processed for immunocytochemistry with a type 1 MyHC specific antibody. Note lack of slow type 1 protein in denervated unstimulated SOL and EDL and presence of such protein in all fibres of denervated stimulated or innervated SOL muscles but in only scattered single or smaller groups of fibres in denervated stimulated or innervated EDL muscles. Sections in this figure are the neighbours of correspondingly labelled sections in Fig. 2. Scale bar = 100 μm.

denervated EDL. However, the amount of slow type 1 MyHC was modest (~10%) and was not increased further by continuing the slow pattern stimulation from 10 to 30 days.

Slow pattern stimulation has the same effect on innervated and denervated EDL muscles

We blocked impulse conduction in the sciatic nerve by TTX and stimulated the fibular nerve to EDL below the block using the same slow stimulus pattern as for the denervated

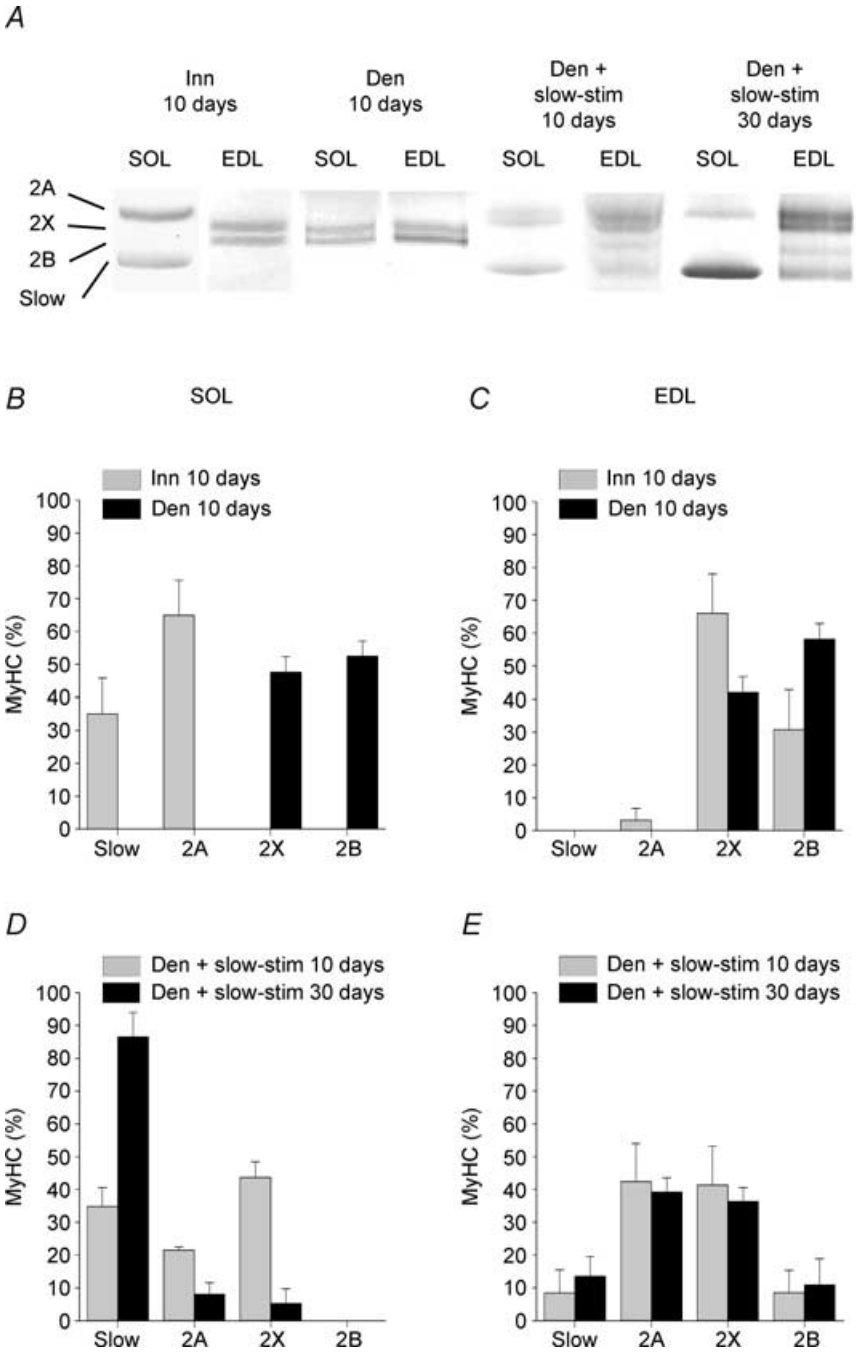

Figure 4. Relative amounts of fibre type specific MyHC proteins in regenerating and differently treated SOL and EDL muscles

Muscles were removed after treatments and at days after intramuscular injections of bupivacaine, as indicated. Muscle homogenates were then examined by SDS-PAGE and Commassie Blue staining (*A*). Relative amounts of protein were quantified densitometrically and plotted as means ± 95% confidence intervals (*B*). $n = 5$ in each experiment.

muscles. In this way, innervated and denervated EDL muscles received identical impulse patterns, which allowed us to find out if the EDL would respond differently to impulse activity transmitted across intact neuromuscular junctions or imposed directly on denervated muscle fibres. As shown in Fig. 5, the effects of nerve stimulation on MyHC mRNA (Fig. 5*A*) and protein (Fig. 5*B* and *C*) expression were the same as those caused by direct stimulation after denervation.

Fast pattern stimulation has the same effect on denervated SOL and EDL

We also stimulated denervated SOL and EDL with a fast, relatively high amount stimulation pattern (60 pulses at 100 Hz every 60 s). After 27 days of such stimulation SOL and EDL contained identical amounts of all fast type 2 and no slow type 1 MyHC (Fig. 6). The expression pattern resembles the default pathway (Fig. 4*B*) except that 2X and 2A expression was now more pronounced and 2B expression correspondingly less pronounced.

Discussion

The present work shows that identical slow pattern stimulation differentially affects MyHC expression in regenerating muscle fibres in SOL and EDL and within EDL of adult rats. After 27 days of such stimulation, SOL expressed 87% and EDL only 13% of slow type 1 MyHC. Satellite cells are generally accepted as the major, and possibly sole, source of new myonuclei in adult muscle. Although other cells types may give rise to new myonuclei, their efficiency in doing so is low and their relevance to normal muscle repair unknown (Zammit *et al.* 2002). Hence, it appears that activated satellite cells in SOL and EDL give rise to muscle fibres that are intrinsically different in their response to a given stimulation pattern.

The stimulation started 3 days after injection of bupivacaine when myotubes in SOL are just beginning to form, functional innervation has not yet been established, and only embryonic and neonatal MyHCs are being expressed (Whalen *et al.* 1990; Grubb *et al.* 1991; Jerkovic *et al.* 1997). By day 3, therefore, newly formed myotubes in SOL and EDL appear already committed to become phenotypically different under identical stimulation. Whether quiescent satellite cells in undamaged SOL and EDL are already so committed or give rise to dividing myoblasts that become so committed before or at the time they form myotubes is unknown.

The slow pattern had the same effect on MyHC expression in EDL whether it was applied directly to denervated fibres or indirectly to innervated fibres by stimulating the nerve below a conduction block by TTX that prevented all endogenous impulse activity from reaching the muscle. Accordingly, the observed effect on MyHC expression was due to the imposed impulse activity *per se* and not to a possible trophic influence from the nerve. This result thus further validates the use of denervated muscles for experiments aimed at clarifying the effects of impulse activity *per se* and at separating these effects from those of putative nerve-derived trophic factors.

Different factors might affect the emergence of fibre types in regenerating muscles. One possibility is that muscle fibres are surrounded by different extracellular matrices, which affect their gene expressions differentially. This possibility has been examined in the chick, where *in vivo* experiments have demonstrated that myoblasts possess intrinsic properties that commit them to develop into different fibre types independent of extrinsic inputs from the mesenchymal stroma (Nikovits *et al.* 2001). Our finding that EDL after 4 weeks of slow pattern stimulation contained scattered slow type 1 fibres points in the same direction. Since many of these type 1 fibres were surrounded by type 2 fibres and therefore largely shared

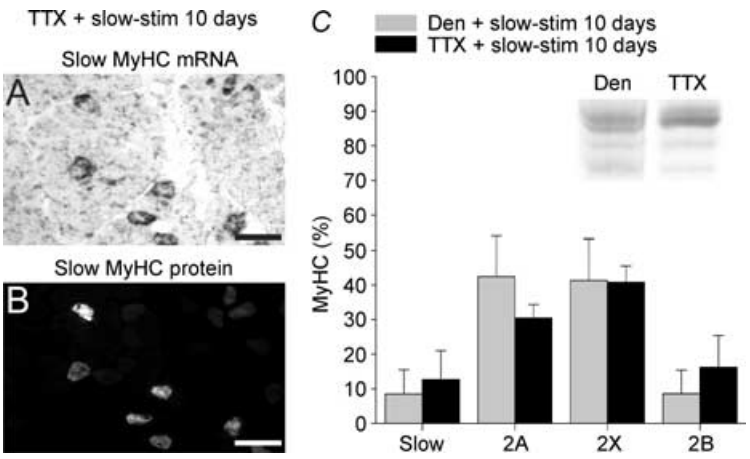

Figure 5. Denervated and innervated EDL muscles respond similarly to slow pattern stimulation
EDL was injected with bupivacaine and either denervated or paralysed by TTX applied locally to the sciatic nerve. Three to 10 days later, the slow stimulus pattern was used to stimulate the denervated EDL directly or the paralysed EDL indirectly through electrodes placed on the fibular nerve below the TTX block. Note scattered fibres positive for MyHC slow transcript (*A*) or protein (*B*) in neighbouring sections from a representative TTX treated and indirectly stimulated EDL muscle, as observed also in denervated and directly stimulated EDL muscles (Figs 2 and 3). The relative amounts of fibre type specific MyHCs were also similar in denervated stimulated and TTX treated and stimulated EDL muscles (*C*, $n = 5$). The histogram representing 10 days denervated and slow-stimulated EDL (shaded) is the same as that in Fig. 4*E* and is shown for comparison. For labelling of protein bands in *C* see Fig. 4. Scale bar: 100 μm.

the same environment, it seems unlikely that differences in the narrow extracellular space between them could be a significant determinant of their different MyHC expression patterns. A second possibility is incomplete muscle fibre necrosis following Marcaine injections if residual segments of the original fibres influence the differentiation of the new fibres that form in continuity with them. However, in longitudinal sections of muscles examined 3 days after multiple injections of Marcaine such segments were few and short and surrounded by mononuclear cells which, presumably, were in the process of invading and destroying them. Moreover, we do not know of any evidence that such segments might determine MyHC expression along the rest of the fibres as they regenerate and become activated by a given pattern of impulses. A third possibility was addressed by Donovan & Faulkner (1987) when they transplanted the fast EDL muscle to the bed of the slow SOL, letting the EDL become reinnervated by the slow SOL nerve but letting it keep its own blood supply in order to prevent muscle fibre necrosis and regeneration. In this situation, innervation by the SOL nerve failed to induce substantial fast to slow transformation of the transplanted EDL, providing evidence against the idea that the position of the EDL in the leg or differences in usage arising from this position are important determinants in MyHC isoform expression, at least in mature non-regenerating muscles. Nevertheless, we cannot rule out that factors such as those just discussed may affect MyHC expression and hence the composition of fibre types in regenerating limb muscles.

A more likely possibility is that regenerating SOL and EDL muscle fibres express different MyHCs during identical stimulation because the satellite cells from which they arise are intrinsically different. Satellite cells from a single mouse limb muscle or even a single muscle fibre are heterogeneous, for example in their expression of myogenic regulatory factors or MyHCs (Rosenblatt *et al.* 1996; Zammit *et al.* 2002). After isolation and culturing *in vitro*, satellite cells from single muscle fibres give rise to myotubes that contain substantial, intermediate, or almost no amount of type 1 MyHC depending on whether the satellite cells come from a single type 1, 2A, or 2B fibre (Rosenblatt *et al.* 1996). Similar differences in amount of type 1 MyHC expression have been obtained for satellite cells cultured and stimulated electrically *in vitro* after isolation from muscles containing large, intermediate, or small numbers of slow type 1 fibres (Wehrle *et al.* 1994). The present results are consistent with such heterogeneity among satellite cells. Not only did regenerating fibres in SOL express much more type 1 MyHC than EDL under identical activation but within EDL the expression was restricted to only a smaller portion of fibres, whose scattered distribution suggested that at least some of them arose from satellite cells in original type 1 fibres.

Snoj-Cvetko *et al.* (1996*a*) let SOL and EDL regenerate in the bed of SOL while innervated by the SOL nerve. After 3 months, type 1 MyHC accounted for more than 80% of total MyHC proteins in the two muscles. In contrast, in the present experiments regenerating EDL expressed strikingly less type 1 MyHCs (13%) than SOL (87%) after 1 month of identical slow pattern stimulation. Since at least 2 months of slow pattern stimulation is needed for transformation of original type 2B or 2X fibres into type 1 fibres in mature non-regenerating EDL muscles (Windisch *et al.* 1998), it is possible that regenerating EDL fibres also require much longer periods of slow pattern stimulation to undergo substantial type 2 to type 1 transformation. If so, the difference between our findings and those of Snoj-Cvetko *et al.* (1996*a*) is due to the different durations of the imposed activity. Furthermore, it is important to note that although type 2B and 2X fibres in the rat EDL may eventually transform into type 1 fibres in terms of MyHC expression, this does not necessarily mean that they also transform into SOL-like type 1 fibres in other respects. For example, after cross-reinnervation of the EDL by the SOL nerve, the twitch contraction time after more than a year is only ∼25 ms, as in normal type 1 muscle fibres of the EDL, whereas it is much longer (∼38 ms) in normal type 1 SOL fibres (Close, 1967, 1969).

Pette *et al.* (2002) exposed the regenerating rat EDL to chronic low frequency stimulation through the nerve. After 30 and 60 days, the EDL contained 34% and 21% type 1

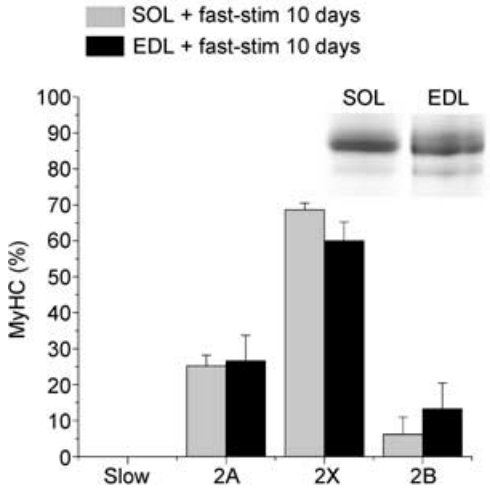

Figure 6. Relative amounts of fibre type specific MyHC protein in denervated SOL and EDL stimulated with a fast stimulus pattern

SOL and EDL were injected with bupivacaine, denervated, and stimulated with a fast stimulus pattern (60 pulses at 100 Hz every 60 s) from days 3 to 30 after the injection and then removed for SDS-PAGE separation of MyHC protein bands (inset) and densitometric analysis of their relative amounts. For labelling of protein bands in inset see Fig. 4. Note that EDL and SOL respond similarly to the fast stimulus pattern. Bars show means ± 95% confidence intervals, $n = 5$.

MyHC, whereas we obtained about 13% type 1 MyHC after 27 days of low frequency stimulation of the denervated EDL. Different stimulation protocols may account for the different quantitative effects. Thus, Pette *et al.* (2002) stimulated continuously at 10 Hz (24 h per day), whereas we stimulated intermittently at 20 Hz (for 10 s every 30 s) to mimic more the natural firing pattern of SOL motor neurones (Hennig & Lømo, 1985). We also started stimulation earlier after Marcaine treatment (day 3 *versus* day 7). Finally, we stimulated the muscle either directly after denervation or indirectly through the nerve in which a proximal TTX block eliminated endogenous impulse activity, whereas such endogenous activity remained in the experiments by Pette and coworkers.

In contrast to the pronounced type 1 MyHC expression in regenerating EDL muscles, Pette *et al.* (2002) found only < 3% type 1 MyHC protein in non-regenerating EDL exposed to the same chronic low frequency stimulation, confirming earlier reports (Ausoni *et al.* 1990; Delp & Pette, 1994). They therefore suggested that chronic low frequency stimulation can directly switch regenerating fibres to a slow programme. Nevertheless, the predominant expression was type 2 MyHC, indicating that many fibres resisted expressing type 1 MyHC during at least 2 months of slow pattern stimulation. Their result is therefore consistent with our conclusion that EDL contains satellite cells that are intrinsically different from those in the SOL with respect to their adaptive potential for type 1 MyHC expression.

Denervated and unstimulated SOL and EDL expressed only fast type 2B and 2X MyHCs (default pathway). The fast pattern stimulation used here had similar effects except that both muscles expressed relatively more 2X MyHC and some 2A MyHC at the expense of type 2B MyHC. We have previously shown that non-regenerating EDL muscles express more 2A/2X MyHC and less 2B MyHC when the amount of high frequency stimulation is increased (Ausoni *et al.* 1990). Since the amount of fast pattern stimulation used in the present experiments was relatively high, the stimulation-induced expression of 2A/2X MyHCs at the expense of 2B MyHC may be similarly explained by an effect of amount *per se*.

In conclusion, the present results show that muscle fibres in adult rat SOL and EDL and within EDL itself express strikingly different patterns of MyHC isoforms as they regenerate during identical slow pattern impulse activation. Thus, different patterns of impulse activity cannot explain the heterogeneity of MyHC expression. Nor did we find any evidence for nerve-derived trophic factors. Other factors related to the extracellular matrix surrounding the muscle fibres, the position and usage of the muscle in the limb, or incomplete muscle fibre necrosis appeared unlikely although they cannot be ruled out. We conclude that our results are best explained by assuming that EDL and SOL contain intrinsically different satellite cells and that the intrinsic properties of these cells together with the pattern of activity imposed on the muscle fibres play a dominant role in determining fibre types in regenerating limb muscles of the rat. Moreover, since also mature non-regenerating SOL and EDL display strikingly different MyHC expression patterns during identical stimulation for at least 2 months (Ausoni *et al.* 1990), both regenerating and non-regenerating SOL and EDL muscle fibres appear to arise from intrinsically different precursor cells. As a result, the descendant fibres of these precursor cells adjust their functional properties to different impulse patterns within intrinsically different adaptive ranges (Westgaard & Lømo, 1988).

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Author's present address

C. Cordonnier: UFR STAPS de l'Université d'Artois, Chemin des Manufactures, 62800 Liévin, France